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From: Gabel, Gailene
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Please provide copy of the following literature:

1) Aoubala, et al., Epitope mapping and immunoactivation of human gastric lipase using five monoclonal antibodies, Eur J Biochem 211 (1-2): 99-104 (1993).

2) Aoubala et al., Immunological technique for the characterization of digestive lipases, Methods Enzymol 286 (Lipases part B), 126-149 (1997).

Thanks a bunch!

Gail Gabel
305-0807
7B15

Ful 1641

butes two variables (the mass of the protein and fraction of the activity) to the equation. One solution for the pH 7 CEH activity data assumed only two components, and suggested that most of the activity was due to a structure considerably larger than that expressing activity at pH 5. This does not mean, however, that a different protein is involved; an oligomer of the pH 5 enzyme is a possibility. This is unlikely, however, given previous experiments involving active site-direted inhibitors, which also pointed to the pH 5 and pH 7 activities being due to different catalytic centers.[19] The target size(s) of the microsomal pH 7 CEH(s) is(are) significantly different than that of the bile salt-dependent neutral CEH (i.e., carboxyl ester lipase) as determined from radiation inactivation target size of the latter enzyme activity as described earlier.

The radiation inactivation results, when taken together with previous observations, are consistent with the suggestion that the microsomal CEH activities are due to distinct enzymes that are different from previously characterized hepatic cholesteryl ester hydrolases such as acid lipase and the bile salt-dependent, neutral CEH. This knowledge, combined with estimates of the sizes of the catalytically active species, is being applied in attempts to solubilize and purify these enzymes.

[7] Immunological Techniques for the Characterization of Digestive Lipases

By MUSTAPHA AOUBALA, ISABELLE DOUCHET, SOFIANE BEZZINE, MICHEL HIRN, ROBERT VERGER, and ALAIN DE CARO

Introduction

In humans, the digestion of dietary triacylglycerols is mediated by two main enzymes, a gastric lipase, which is secreted in the upper part of the digestive system and acts along the whole gastrointestinal tract, and a pancreatic lipase, which contributes to lipid digestion only in the duodenum.[1-3] Moreover, to overcome the inhibitory effects of the bile salts present in the intestinal lumen, pancreatic lipase specifically requires the presence of a small pancreatic cofactor (colipase), which acts as an anchor for pancreatic

[1] Y. Gargouri, H. Moreau, and R. Verger, *Biochim. Biophys. Acta* **1006**, 255 (1989).
[2] M. Hamosh, "Lingual and Gastric Lipases: Their Role in Fat Digestion," CRC Press, Boca Raton, Florida, 1990.
[3] F. Carrière, J. A. Barrowman, R. Verger, and R. Laugier, *Gastroenterology* **105**, 876 (1993).

0076-6879/97 $25.00

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OUCHET, SOFIANE BEZZINE,
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lipase on bile salt-coated lipid interfaces.[4] Gastric and pancreatic lipases belong to two distinct groups in terms of their primary structure and biochemical properties. The group I family includes pancreatic lipase, lipoprotein lipase, and hepatic lipase.[5] These enzymes have been shown to share common characteristics, such as a molecular weight ranging from 50,000 to 55,000, a high homology of their primary structures (60%), and an optimal activity expressed at alkaline pH. The group II family, defined as the acidic lipase family,[6–8] includes preduodenal lipases and lysosomal lipase and shares no sequence homology with the pancreatic lipase family.

To gain a better understanding of the physiologic functions and structural characteristics of the digestive lipases, human pancreatic lipase (HPL) and human grastric lipase (HGL), monoclonal antibodies (mAbs) may provide useful tools. Such antibodies would also be useful for identification and purification of the enzymes from biological fluids. In this chapter, we describe the preparation and possible application of polyclonal (pAb) and mAbs to HGL and HPL obtained from gastric and pancreatic juices, respectively.

Production and Purification of Monoclonal and Polyclonal Antibodies to Human Gastric and Pancreatic Lipases

Preparation of Antigens

Human Gastric Lipase. HGL is purified to homogeneity from gastric juice[9] from volunteers with the sequential use of chromatographies on Mono S (sulfopropyl cation exchanger, Pharmacia, Uppsala, Sweden) and Mono Q (diethyl aminoethyl anion exchanger, Pharmacia).

Human Pancreatic Lipase. HPL is purified to homogeneity from pancreatic juice according to De Caro *et al.*[10]

[4] C. Erlanson-Albertsson, *Biochim. Biophys. Acta* **1125,** 1 (1992).
[5] S. B. Petersen, and F. Drabløs, *in* "Lipases: Their Biochemistry, Structure and Application" (P. Woolley and S. Petersen, eds.), p. 23. Cambridge University Press, Cambridge, England, 1994.
[6] R. A. Anderson and G. N. Sando, *J. Biol. Chem.* **266,** 22479 (1991).
[7] D. Ameis, M. Merkel, C. Eckerskorn, and H. Greten, *Eur. J. Biochem.* **219,** 905 (1994).
[8] F. Carrière, Y. Gargouri, H. Moreau, S. Ransac, E. Rogalska, and R. Verger, *in* "Lipases: Their Structure, Biochemistry and Application" (P. Wooley and S. B. Petersen, eds.), p. 181. Cambridge University Press, Cambridge, England, 1994.
[9] H. Moreau, C. Abergel, F. Carrière, F. Ferrato, J. C. Fontecilla-Camps, C. Cambillau, and R. Verger, *J. Mol. Biol.* **225,** 147 (1992).
[10] A. de Caro, C. Figarella, J. Amic, R. Michel, and O. Guy, *Biochim. Biophys. Acta* **490,** 411 (1977).

Production of Monoclonal Antibodies by Hybridomas

Two young female BALB/c mice were immunized for each antigen with HGL or HPL as follows: the first and the second immunizations were carried out with 100 μg of antigen suspended in 100 μl of 10 m*M* phosphate buffer (pH 7.4), containing 150 m*M* sodium chloride (PBS), emulsified in 100 μl of complete Freund's adjuvant (200 μl final volume), and injected subcutaneously at 10-day intervals. Two weeks after the second injection, the mice were bled and the serum tested in a direct binding enzyme-linked immunosorbent assay (ELISA) as described later. The mouse with the highest titre was selected and 100 μg of pure antigen, suspended in 200 μl of PBS, was again injected subcutaneously. Ten days later, three fusion-priming intraperitoneal injections of 50 μg of HGL or HPL each in PBS were given on 3 subsequent days. The fusion procedure followed is that described by Galfré *et al.*[11] and Kohler and Milstein.[12]

Spleen was harvested 1 day after the last injection and a spleen cell suspension prepared by gently teasing the spleen while holding it with a pair of forceps in a petri dish and fused with nonsecreting mouse myeloma P3 X63 Ag8. 653 (ATCC).[12,13] Cell fusion was performed using polyethylene glycol 1500 (Boehringer Mannheim, Mannheim, Germany). The hybridomas in hypoxanthine-aminopterin-thymidine (HAT) medium were seeded into six microtiter tissue culture plates containing murine peritoneal macrophages as a feeder layer. Ten days after the fusion, 180 μl of culture supernatant was removed from each well to test the antilipase activity in a direct-binding ELISA test.

ELISA Tests for Screening Antilipase MAbs

When not stated otherwise, the following buffers were used for all ELISA procedures. Coating buffer: PBS. Wash medium: PBS containing bovine serum albumin (BSA) (5 g/liter) and Tween-20 (0.5 g/liter). Saturating buffer: PBS containing BSA (5 g/liter). Substrate solution: *o*-phenylenediamine (Sigma, St. Louis, MO) (0.4 g/liter) in 0.05 *M* sodium phosphate/citrate, pH 5, containing fresh hydrogen peroxide (0.4%). Stop solution: 1 *M* sulfuric acid.

The screening of hybridoma supernatants was carried out by performing solid phase immuno assays using 96-well microtiter polyvinyl chloride (PVC) plates (Maxisorb, Nunc, Roskilde, Denmark). For screening anti-HGL mAbs, a simple sandwich ELISA was carried out by coating the

[11] G. Galfré, S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard, *Nature* **266,** 550 (1977).
[12] G. Kohler and C. Milstein, *Nature* **256,** 495 (1975).
[13] J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewki, *J. Immunol.* **123,** 1548 (1979).

Hybridomas

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nd J. C. Howard, *Nature* **266,** 550 (1977).

Rajewki, *J. Immunol.* **123,** 1548 (1979).

plates with 500 ng of pure native HGL per well, in 50 μl coating buffer, overnight at 4°.[14] For screening anti-HPL mAbs, a double sandwich ELISA was performed by coating the plates with 500 ng of pure anti-HPL pAb per well (captor antibody).[15] After saturation of the remaining free sites with saturating buffer (200 μl per well) for 2 hr at room temperature, HPL was added to the PVC-coated pAb (500 ng per well), which, unlike the simple sandwich ELISA, results in the random orientation of the various epitopic regions of HPL. Then hybridoma supernatants (50 μl) were added to each well and incubated for 1 hr at room temperature. mAb-producing hybridomas were detected with peroxidase-conjugated antimouse IgG antibody (Sigma). The substrate solution of peroxidase was used to quantify the positive clones. The reaction was stopped with a 1 *M* sulfuric acid solution (50 μl per well) and the optical density (OD) was read on an automatic plate reader (Dynatech, Guernsey, UK) at 492 nm. Between each step in the assay, the plates were rinsed three times with wash medium.

Four anti-HPL mAbs (81-23, 146-40, 315-25, and 320-24) were found to react with the simple sandwich ELISA, while mAb 248-31 did not react.[15] All five antibodies, however, interacted with HPL in the double sandwich ELISA. These results can be explained by the fact that the epitope recognized by mAb 248-31 is in a hydrophobic region adsorbed to the PVC plate and is therefore not accessible to the antibody. An alternative explanation might be that a conformational change (denaturation) may have occurred during HPL adsorption to the PVC plate, resulting in the loss of the recognition site. In the case of proteins with functional hydrophobic regions such as lipolytic enzymes, the double sandwich ELISA test involving specific polyclonal antibodies adsorbed as the first layer on PVC plates therefore yields randomly oriented antigenic regions and preserves the second antibody recognition.

MAbs Isotype Identification

A mouse monoclonal antibody isotyping kit (Amersham, Buckinghamshire, UK) was used to determine the antibody class of the various antilipase mAbs. The isotypes of mAbs were set up according to the manufacturer's instructions, with culture supernatants of each clone after limiting dilutions. All the tested mAbs belong to the IgG_1 class with a κ light chain with the exception of the anti-HGL mAb 13-42 and the anti-HPL mAb 248-31, which are of the IgG_{2b} isotype.

[14] M. Aoubala, C. Daniel, A. de Caro, M. G. Ivanova, M. Hirn, L. Sarda, and R. Verger, *Eur. J. Biochem.* **211,** 99 (1993).

[15] M. Aoubala, L. de la Fournière, I. Douchet, A. Abousalham, C. Daniel, M. Hirn, Y. Gargouri, R. Verger, and A. de Caro, *J. Biol. Chem.* **270,** 3932 (1995).

Purification of MAbs

Positive hybridomas were cloned using the limiting dilution technique. For ascites production, 2.5×10^6 hybridoma cells were injected intraperitoneally into BALB/c mice. mAbs were purified from mouse ascitic fluids by precipitation with 50% saturated ammonium sulfate. The protein solution was loaded onto a Protein A-Sepharose CL-4B column (Pharmacia), previously equilibrated with 20 m*M* borate buffer, pH 8.8, containing 2.5 *M* sodium chloride. Then the column is extensively washed with the same buffer and the antibodies eluted with 50 m*M* sodium citrate, pH 4.5 or 5.0, for antibodies of the IgG_{2b} or IgG_1 subclasses, respectively. Fractions containing antibodies were dialyzed against PBS containing 0.02% sodium azide, concentrated to 4 mg/ml using Centriprep 30 concentrators (Amicon, Beverly, MA), filtered through 0.22-μM Millipore filters and stored at −20° in small aliquots. The purity of the IgG preparations was checked by SDS–PAGE (7.5%).

Preparation of Anti-HGL and Anti-HPL Polyclonal Antibodies

Rabbits were immunized with native HGL according to the following schedule: 1 mg of HGL in 0.5 ml Freund's complete adjuvant (ICN ImmunoBiologicals, Costa Mesa, CA) was injected subcutaneously on day 0. Three weeks later, 1 mg of HGL, in 0.5 ml Freund's incomplete adjuvant (ICN ImmunoBiologicals), was injected intramuscularly in the footpads every 10 days for 1 month. The last injection included 0.5 mg HGL in PBS and was given intravenously in the ear 1 week before the animals were sacrificed. One week after each booster injection, sera were tested for anti-HGL reactivities by performing ELISA as described earlier.

The rabbit anti-HGL pAb was purified using a column of immobilized HGL. For that purpose, 16 mg of purified HGL was immobilized on 7 ml of swollen Affi-Gel 10 (Bio-Rad, Hercules, CA) equilibrated with PBS. After 4 hr of incubation at 4° and under agitation, unreacted gel groups were blocked with a solution of 0.5 *M* glycinamide (Sigma) (pH 7.5). The gel was poured into a glass column (1.5 × 3.5 cm) and washed successively with 50 ml of 0.2 *M* glycine-HCl buffer (pH 2.2) and 100 ml of PBS. Under these conditions, more than 84% of the initial amount of HGL was covalently coupled to the gel. For the purification of specific anti-HGL pAb, rabbit anti-HGL sera were first precipitated with 50% saturated ammonium sulfate. After centrifugation at 10,000*g* for 30 min, the pellet was solubilized in 25 m*M* Tris-HCl (pH 7.4) containing 150 m*M* NaCl (TBS), dialyzed against the same buffer and loaded on the HGL-Affi-Gel 10 column previously equilibrated with TBS. After 4 hr of incubation at 4°, the column was washed successively with TBS, with 25 m*M* Tris-HCl (pH 7.4) con-

g the limiting dilution technique.
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Polyclonal Antibodies

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intramuscularly in the footpads
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30 min, the pellet was solubilized
150 m*M* NaCl (TBS), dialyzed
HGL-Affi-Gel 10 column pre-
of incubation at 4°, the column
25 m*M* Tris-HCl (pH 7.4) con-

taining 0.5 *M* NaCl and 0.2% Triton X-100 and then again with the initial TBS until zero absorbance at 280 nm was reached. Pure antibodies directed against HGL were eluted with a 0.2 *M* glycine-HCl buffer (pH 2.4). Each fraction (1 ml) was immediately neutralized with 250 μl of 1 m*M* Tris-HCl (pH 9.0); then the fractions containing antibodies were pooled, dialyzed against PBS, and concentrated to about 3 mg/ml using an Amicon ultrafiltration cell. They are stored at −20° in small aliquots. These antibodies gave a single band on SDS–PAGE.

Characterization and Application of Monoclonal Antibodies

Monoclonal antibodies that have been produced against HGL and HPL are listed in Tables I and II. The mAbs are characterized in terms of affinity to their antigen, capacity to inhibit lipase activity or to bind lipids, epitope mapping, and cross-reactivity with other gastric or pancreatic lipases. These antibodies constitute useful tools for the study of mammalian acidic and pancreatic lipases.

Immunoinactivation Studies

To locate some epitopes at or near the catalytic site of HGL, we tested the ability of each mAb to inhibit lipase activity.[14] A fixed amount of

TABLE I
CHARACTERISTICS OF mAbs TO HGL

	mAb 4.3	mAb 13.42	mAb 25.4	mAb 35.2	mAb 53.27	mAb 83.15	mAb 218.13
Affinity constant ($\times 10^{-7}\ M^{-1}$)	1.03	ND[a]	1.58	0.19	ND	0.02	8.80
Inhibition of enzymatic activity[b]	Yes	ND	Yes	Yes	ND	Yes	No
Inhibition of lipid binding (monolayer)[c]	Yes	ND	Yes	Yes	ND	Yes	No
Reactivity of HGL by simple sandwich ELISA[d]	+	+	+	+	+	++	+++
Reactivity of HGL by Western blot[e]	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Cross-reactivity to other preduodenal lipases by Western blot							
DGL	Yes	ND	Yes	No	ND	No	No
RGL	Yes	ND	Yes	Yes	ND	No	No

[a] ND, Not determined.
[b] From Aoubala *et al.*[14]
[c] From Ivanova *et al.*[19]
[d] See Fig. 2.
[e] See Fig. 4.

TABLE II
CHARACTERISTICS OF mAbs TO HPL

	mAb 81.23	mAb 146.40	mAb 248.31	mAb 315.25	mAb 320.24
Affinity constant ($\times 10^{-9}$ M^{-1})	7.0	1.9	ND[a]	6.0	4.5
Inhibition of enzymatic activity	Yes	No	Yes	Yes	Yes
Reactivity of HPL by simple sandwich ELISA	+	+	−	+	+
Reactivity of HPL by double sandwich ELISA	+	+	+	+	+
Reactivity of native HPL by Western blot	Yes	Yes	Yes	Yes	Yes
Reactivity of SDS-denatured HPL by Western blot	Yes	Yes	No	Yes	Yes
Cross-reactivity to other pancreatic lipases by Western blot					
Porcine pancreatic lipase	No	No	No	No	No
Dog pancreatic lipase	No	No	No	No	No
Horse pancreatic lipase	Yes	No	No	Yes	Yes
Guinea pig pancreatic lipase	No	No	No	No	No

[a] ND, Not determined.

enzyme (7–30 μg) was incubated with each mAb at various molar ratios (mAb/HGL: 0.5–2). Incubations were performed in PBS (20-μl final volume) for 1 hr at 37°. The residual activity of the HGL–mAb complexes was determined using three substrates differing in their fatty acid chain length: tributyrin and soybean oil emulsions as well as a substrate-forming stable film: 1,2-didecanoyl-*sn*-glycerol (dicaprin). Assays on tributyrin (Fluka) and soybean oil (commercial grade) emulsified in gumarabic were carried out with the bulk pH-stat method under standard conditions previously described by Gargouri *et al.*[16] The kinetics of the hydrolysis of 1,2-didecanoyl-*sn*-glycerol (SRL) films by HGL[17] were recorded, with or without incubation of the enzyme with each mAb, using the barostat technique previously described by Verger and de Haas.[18]

Three classes of mAbs have been identified on the basis of their inhibitory effect: class 1 includes three mAbs (4-3, 25-4, and 35-2) possessing the capability to inhibit HGL on all the substrates tested. The second class, consisting of mAb 83-15, preferentially prevents the lipolytic activity of HGL on long-chain triacylglycerols. Last, mAb 218-13, which belongs to class 3, had no inhibitory effect (Table I).

[16] Y. Gargouri, G. Piéroni, C. Rivière, J.-F. Saunière, P. A. Lowe, L. Sarda, and R. Verger, *Gastroenterology* **91,** 919 (1986).
[17] Y. Gargouri, G. Pièroni, F. Ferrato, and R. Verger, *Eur. J. Biochem.* **169,** 125 (1987).
[18] R. Verger and G. H. de Haas, *Chem. Phys. Lipids* **10,** 127 (1973).

Abs TO HPL

nAb	mAb	mAb	mAb	mAb
1.23	146.40	248.31	315.25	320.24
7.0	1.9	ND[a]	6.0	4.5
Yes	No	Yes	Yes	Yes
+	+	−	+	+
+	+	+	+	+
Yes	Yes	Yes	Yes	Yes
Yes	Yes	No	Yes	Yes
No	No	No	No	No
No	No	No	No	No
Yes	No	No	Yes	Yes
No	No	No	No	No

ch mAb at various molar ratios
rformed in PBS (20-μl final vol-
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ffering in their fatty acid chain
ns as well as a substrate-forming
dicaprin). Assays on tributyrin
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he kinetics of the hydrolysis of
HGL[17] were recorded, with or
ch mAb, using the barostat tech-
l de Haas.[18]
tified on the basis of their inhibi-
-3, 25-4, and 35-2) possessing the
strates tested. The second class,
revents the lipolytic activity of
mAb 218-13, which belongs to

, P. A. Lowe, L. Sarda, and R. Verger,

r, *Eur. J. Biochem.* **169,** 125 (1987).
10, 127 (1973).

Taking advantage of the low surface activity of IgG molecules,[19] the capacity of the mAbs to inhibit the penetration of native and sulfhydryl-modified HGL (Nbs-HGL) into dicaprin monolayers was investigated using the monomolecular film technique.[19] The HGL–mAb and Nbs-HGL–mAb complexes (molar ratio = 0.5) were therefore obtained by incubating each mAb with HGL or Nbs-HGL exactly as described earlier. All the inhibitory mAbs reduce the lipid-binding capacity of HGL and only the noninhibitory mAb 218-13 does not affect the penetration properties of the enzyme. We have not yet detected any inhibitory mAb that does not affect the lipase penetration. With these experimental data it is not possible to distinguish which of the two extreme situations holds true: the catalytic site and lipid-binding domain are either spatially very close or extremely far apart from each other. Based on the known three-dimensional structures of four lipases,[20–23] it is probable that the two functionally distinct sites of HGL are topographically close.

The potentially inhibitory effects of the five anti-HPL mAbs were also tested.[15] Four mAbs (81-23, 248-31, 315-25, and 320-24) reduced the hydrolysis of trioctanoin emulsion by HPL, whereas mAb 146-40 had no effect (see Table II). It is worth noting that comparable inhibition levels were observed in both the presence and absence of colipase and bile salts, probably because the affinity of HPL for mAb is higher than that for the lipids.[15]

Mapping of Epitopes on the Surface of Native Lipases

To test whether the different mAbs recognized different epitopes on the enzyme, the ELISA double-antibody-binding test (ELISA additivity test) initially developed by Friguet *et al.*[24] was used. Each mAb was first titrated by performing a direct ELISA test in which increasing amounts of each mAb (0.125–2 μg per well) were incubated with a fixed amount of lipase (2–5 ng per well) previously coated on the microplate. The ELISA experiments were performed as described earlier for the screening of mAbs. For each mAb, a titration curve was established, and the mAb concentration

[19] M. G. Ivanova, M. Aoubala, A. de Caro, C. Daniel, J. Hirn, and R. Verger, *Colloids Surf.* **B1,** 17 (1993).
[20] L. Brady, A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, and U. Menge, *Nature* **343,** 767 (1990).
[21] F. K. Winkler, A. d'Arcy, and W. Hunziker, *Nature* **343,** 771 (1990).
[22] J. D. Schrag, Y. Li, S. Wu, and M. Cygler, *Nature* **351,** 761 (1991).
[23] C. Martinez, P. de Geus, M. Lauwereys, G. Matthyssens, and C. Cambillau, *Nature* **356,** 615 (1992).
[24] B. Friguet, A. F. Chaffotte, L. Djavadi-Ohaniance, and M. E. Goldberg, *J. Immunol. Meth.* **77,** 305 (1985).

that gives the maximal signal corresponds to the saturation of all the accessible epitopes. This mAb concentration was then used for the cotitration of the mAbs in pairs.

Two specific mAbs were mixed prior to incubation with lipase adsorbed to the PVC plate and the competition between antibodies for the antigen was expressed by means of additivity indexes.[24] The different mAbs anti-HGL and anti-HPL were studied in all possible pairs and the additivity index (AI), which characterizes each pair of specific antibodies, was determined using the following formula:

$$\mathrm{AI} = 100 \cdot \left(\frac{2 \cdot A_{1+2}}{A_1 + A_2} - 1 \right),$$

where A_{1+2} is the absorbance obtained in the ELISA with the mixture of two mAbs at a 1 : 1 molar ratio, and A_1 and A_2 are the absorbances obtained with each mAb, respectively. This index (expressed as a percentage) makes it possible to evaluate the simultaneous binding of two mAbs to the antigen. According to Friguet *et al.*,[24] if the two mAbs have the same specificity and bind randomly at the same antigenic site, A_{1+2} will be equal to the mean value of A_1 and A_2, and then AI will be equal to zero (competitive binding). Conversely, if the two mAbs bind independently at two different antigenic sites (additive binding), A_{1+2} will be equal to the sum of A_1 and A_2 and then AI will be equal to 100. Values of AI ranging from 0 to 100 will indicate that the two mAbs bind to closely associated overlapping epitopes (partially additive binding).

From this method, we found that the various epitopes of HGL are partially overlapping and are all located in the same antigenic region.[14] For HPL, the mAbs can be classified into two groups of antibodies directed against two different antigenic determinants: group I includes three mAbs (81-23, 315-25, and 320-24) and group II consists of mAb 146-40.[15] Because mAb 248-31 did not react in a simple ELISA, it was not possible to localize its epitope by this technique.

Mapping of Epitopes along the Primary Sequence of Lipases

An epitope mapping study was also performed using the peptide mapping method described by Wilson and Smith.[25] Briefly, HGL and HPL were subjected to limited digestion with trypsin[26] and chymotrypsin,[27] respec-

[25] J. E. Wilson and A. D. Smith, *J. Biol. Chem.* **260,** 12838 (1985).

[26] M. Aoubala, J. Bonicel, C. Bénicourt, R. Verger, and A. de Caro, *Biochim. Biophys. Acta* **1213,** 319 (1994).

[27] A. Abousalham, C. Chaillan, B. Kerfelec, E. Foglizzo, and C. Chapus, *Protein Eng.* **5,** 105 (1992).

lo the saturation of all the accessi-
s then used for the cotitration of

) incubation with lipase adsorbed
tween antibodies for the antigen
exes.[24] The different mAbs anti-
possible pairs and the additivity
of specific antibodies, was deter-

$$\left(\frac{A_{1+2}}{A_2} - 1\right),$$

the ELISA with the mixture of
A_2 are the absorbances obtained
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bs have the same specificity and
A_{1+2} will be equal to the mean
al to zero (competitive binding).
dently at two different antigenic
l to the sum of A_1 and A_2 and
AI ranging from 0 to 100 will
associated overlapping epitopes

various epitopes of HGL are
the same antigenic region.[14] For
o groups of antibodies directed
ts: group I includes three mAbs
nsists of mAb 146-40.[15] Because
A, it was not possible to localize

equence of Lipases

rformed using the peptide map-
1.[25] Briefly, HGL and HPL were
n[26] and chymotrypsin,[27] respec-

12838 (1985).
nd A. de Caro, *Biochim. Biophys. Acta*

lizzo, and C. Chapus, *Protein Eng.* 5,

tively. The products were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose or Glassybond membranes for Western blotting and *N*-terminal amino acid sequence analysis, respectively.

The results of the immunoblotting experiments carried out with the tryptic HGL fragments showed that anti-HGL mAbs can be divided into two groups.[26] The first group comprises five mAbs (4-3, 13-42, 25-4, 35-2, and 83-15), which recognize only the epitopes located in the *N*-terminal domain of HGL. The second group contains two mAbs (53-27 and 218-13), which immunoreact only with the *C*-terminal domain of HGL (Fig. 1A).

For HPL, four fragments resulting from the limited chymotryptic cleavage of HPL (14, 26, 30, and 36 kDa) were characterized.[15] MAb 146-40

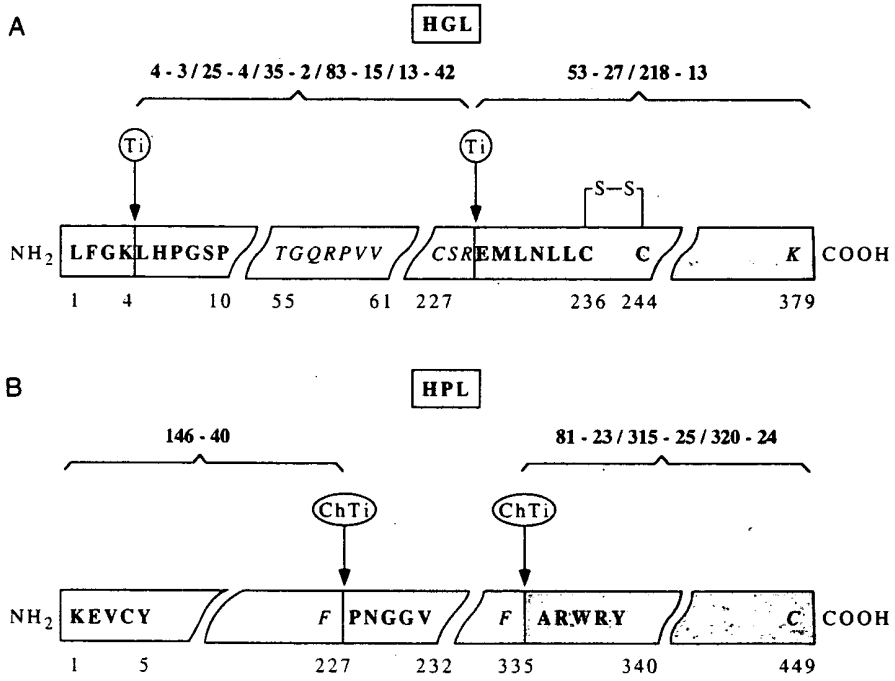

FIG. 1. (A) Schematic representation of the primary sequence of HGL adapted from Bodmer *et al.*[35] showing the tryptic cleavage sites (Ti) and the *N*-terminal domain (empty frame) as well as the *C*-terminal domain (shaded frame). (B) Diagram of the primary sequence of HPL from Winkler *et al.*[21] The arrow shows the chymotryptic cleavage sites (ChTi). The *N*-terminal domain (empty frame) as well as the *C*-terminal domain (shaded frame) are indicated. In both representations, amino acid sequences, determined using the Edman degradation technique, are indicated in boldface letters and those deduced from the cDNA sequence are indicated in italic letters. Numbering over brackets indicates the mAbs that recognize their corresponding fragments.

reacted with the 30- and 36-kDa *N*-terminal fragments, indicating that the epitope recognized by mAb 146-40 is located in the fragment (Lys-1–Phe-227) (Fig. 1B). In the same study we have reported that three mAbs (81-23, 315-25, and 320-24) did not immunoreact with the *C*-terminal domain (Ala-336–Cys-449, 14 kDa). More recently, we observed[28] that when HPL was submitted to an extensive chymotryptic cleavage, a positive reactivity of these three mAbs with the 14-kDa fragment. Our initial results[15] can be explained by an insufficient chymotryptic proteolysis generating low amounts of the *C*-terminal domain. We can now conclude that epitopes recognized by mAbs 81-23, 315-25, and 320-24 are located in the *C*-terminal domain (Ala-336–Cys-449) (Fig. 1B).

Discrimination of the Pancreatic Lipase Structural Domains by MAbs

We also determined the immunoreactivity of anti-HPL mAbs toward three variants of HPL: N-HPL, HPL(-lid), and a N-GPLRP2/C-HPL chimera, produced in insect cells using the baculovirus expression system.[29] N-HPL consists of the *N*-terminal domain of HPL only (Lys-1–Phe-335). HPL(-lid) has a mini-lid of 5 amino acid residues, instead of 23 in HPL (Cys-237–Cys-261), and the chimera is made of the *N*-terminal domain of the guinea pig pancreatic lipase related protein 2 (GPLRP2) and the *C*-terminal domain of HPL.

Western blot analysis of these different mutants showed that the 146-40 mAb recognized HPL, HPL(-lid), N-HPL but neither GPLRP2 nor the N-GPLRP2/C-HPL chimera nor the purified *C*-terminal domain of HPL. These results allowed us to conclude that the epitope recognized by the mAb 146-40 is located within the *N*-terminal domain of HPL but not in the lid (Cys-237–Cys-261). Moreover, the 81-23, 315-25, and 320-24 mAbs recognized HPL, HPL(-lid), the N-GPLRP2/C-HPL chimera, and the purified *C*-terminal domain of HPL but neither N-HPL nor GPLRP2. We confirmed that these three mAbs are directed against the *C*-terminal domain of HPL.

Cross-Species Reactivity of Anti-HGL MAbs

Immunological cross-reactivity of the various anti-HGL mAbs with DGL, RGL, and HPL as a control experiment was undertaken by using three different techniques: (1) a simple sandwich ELISA, (2) an indirect competitive assay between two lipases for the same antibody, and (3) the Western blotting technique using SDS-denatured lipases.

[28] S. Bezzine, F. Carrière, J. De Caro, R. Verger, and A. De Caro, in preparation (1997).
[29] F. Carrière, K. Thirstrup, S. Hjorth, F. Ferrato, P. F. Nielsen, C. Withers-Martinez, C. Cambillau, E. Boel, L. Thim, and R. Verger, *Biochemistry* **36,** 239 (1997).

ıinal fragments, indicating that the ıcated in the fragment (Lys-1–Phe- /e reported that three mAbs (81-23, :t with the *C*-terminal domain (Ala- we observed[28] that when HPL was c cleavage, a positive reactivity of ıgment. Our initial results[15] can be ryptic proteolysis generating low e can now conclude that epitopes 320-24 are located in the *C*-terminal

Structural Domains by MAbs

activity of anti-HPL mAbs toward id), and a N-GPLRP2/C-HPL chi- e baculovirus expression system.[29] ain of HPL only (Lys-1–Phe-335). id residues, instead of 23 in HPL made of the *N*-terminal domain of l protein 2 (GPLRP2) and the *C*-

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he various anti-HGL mAbs with ıeriment was undertaken by using sandwich ELISA, (2) an indirect or the same antibody, and (3) the lenatured lipases.

and A. De Caro, in preparation (1997).
), P. F. Nielsen, C. Withers-Martinez, C. *ochemistry* **36,** 239 (1997).

For the simple sandwich ELISA, microtitration plates were coated with 50 ng of each of the pure lipase and incubated with a fixed amount of anti-HGL mAb (500 ng/well). The ELISA procedure was performed as described earlier. Figure 2 shows the cross-reactivity of each mAb with native lipases. None of the five mAbs reacts with HPL. This result is in good agreement with the fact that there is no sequence homology between gastric and pancreatic lipases. These mAbs, however, showed different binding properties toward HGL, DGL, and RGL. Two mAbs (4-3 and 25-4) were found to react with these three lipases. These results show that the epitopes recognized by these two mAbs are conserved for the three enzymes. In contrast, mAbs 35-2 and 83-15 do not show significant reactivity with DGL and RGL adsorbed on the PVC plate. Finally mAb 218-13 presents a significant cross-reactivity with DGL.

Since these three gastric lipases present a high-sequence identity (about 86%), the absence of a cross-reactivity between some anti-HGL mAbs and DGL and RGL could be explained by the fact that the epitopes recognized by these antibodies are not conserved on the surface of these enzymes. An alternative explanation could be that the epitopes are hidden or denatured when the enzymes are adsorbed to the PVC plate.

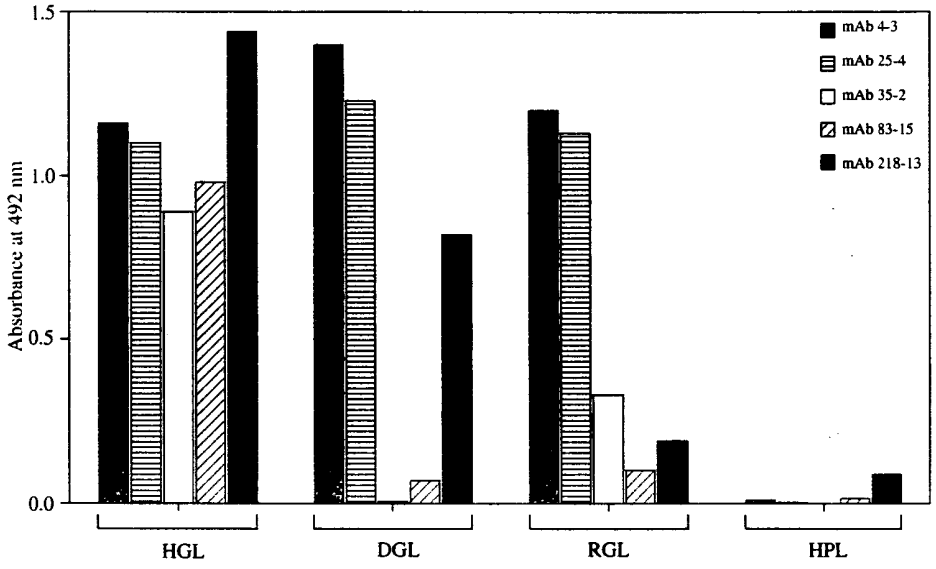
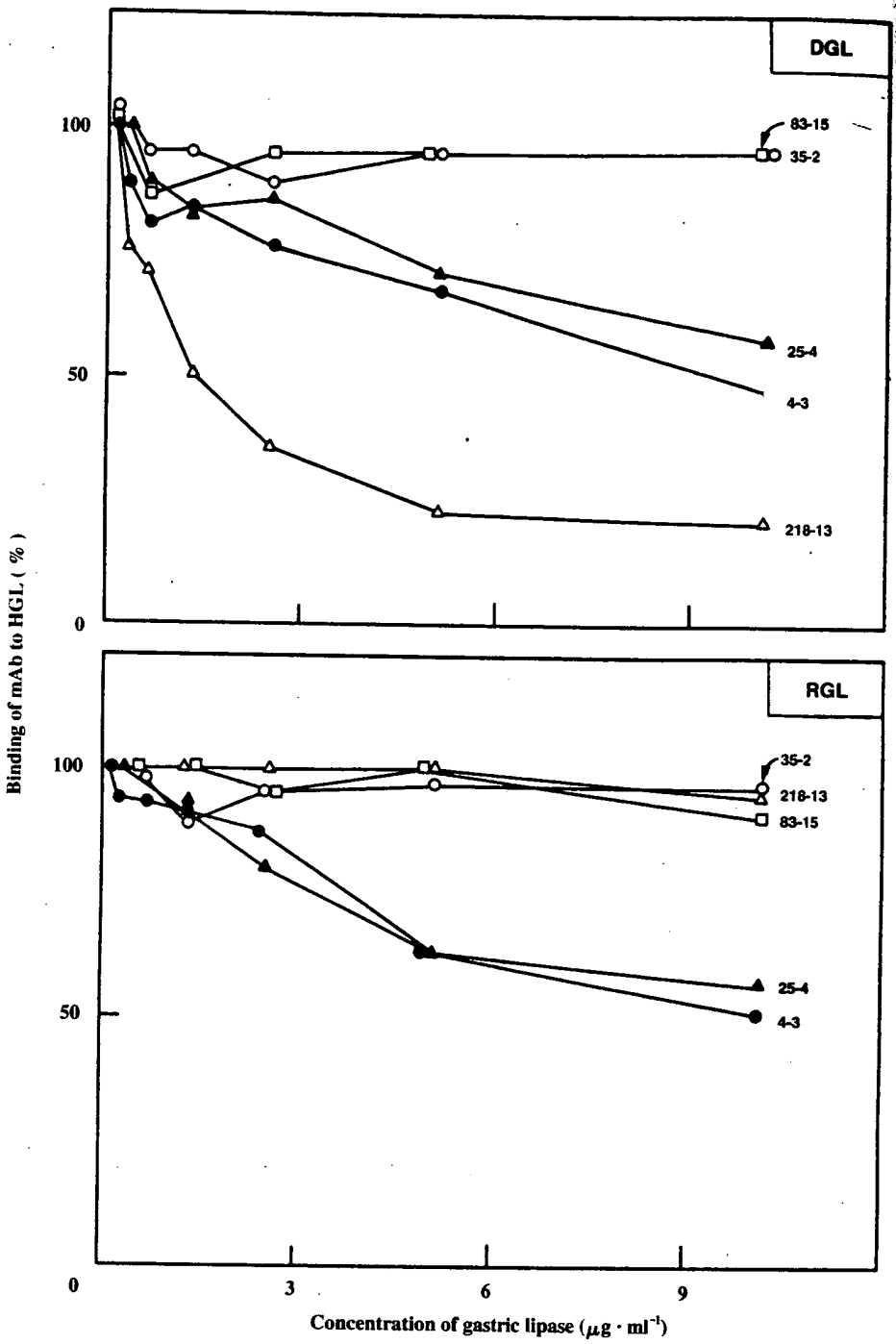
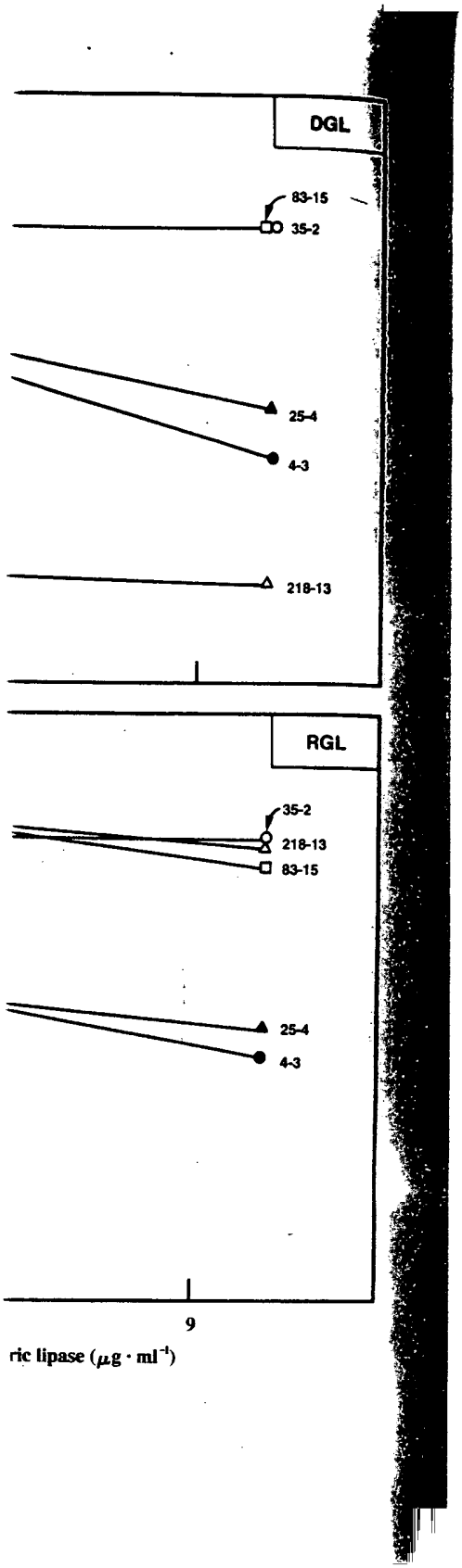


FIG. 2. Cross-species reactivity of anti-HGL mAbs with gastric lipases from human (HGL), dog (DGL), rabbit (RGL), and HPL using a simple sandwich ELISA. The assay was performed by direct coating of the various lipases (50 ng/well) with a constant amount of anti-HGL mAb (500 ng/well).


DGL
100
50
0
83-15
35-2
25-4
4-3
218-13
RGL
100
50
0
35-2
218-13
83-15
25-4
4-3
3
6
9
Binding of mAb to HGL (%)
Concentration of gastric lipase (μg · ml⁻¹)



The immunological cross-reactivity of anti-HGL mAbs was also studied using an indirect competitive assay. This technique allowed us to study the interaction between the different mAbs with the antigens in solution. For that purpose, DGL or RGL at various concentrations (0.005–10 μg/ml) was mixed with a constant amount of each mAb (2 μg/ml) in PBS (200-μl final volume). The mixtures were incubated for 2 hr at 37° and then overnight at 4°. After this incubation the presence of unbound mAbs was quantified by performing a simple sandwich ELISA. For that, 100 μl of each mixture was incubated for 2 hr at 37° into the wells of a microtitration plate, previously coated with HGL (20 ng/well). The ELISA procedure was the same as that described earlier. The results (see Fig. 3) confirm that the epitopes recognized by mAbs 4-3 and 25-4 are conserved on the surface of these gastric lipases and correspond to highly conserved structural elements. The epitope of mAb 218-13 is also well conserved only in DGL. In contrast, the epitopes of mAbs 35-2 and 83-15 are not conserved in DGL and RGL.

The reactivity of the preceding mAbs with SDS-denatured lipases was also studied by Western blotting. Results are presented in Fig. 4. First, note that all the anti-HGL–mAbs immunoreact with SDS-denatured HGL, suggesting that the epitopes recognized by these mAbs are probably continuous determinants (see Fig. 4 and Table I). Second, mAbs 4-3 and 25-4 immunoreact with SDS-denatured DGL and RGL. In contrast, mAbs 35-2 and 83-15 show no detectable reactivity toward SDS-denatured DGL, confirming the previous results obtained with the ELISA tests (Figs. 2 and 3).

In contrast, mAb 218-13 loses its immunoreactivity on SDS-denatured DGL (Fig. 4) as compared with the native DGL (see Figs. 2 and 3). These results show that the epitope of mAb 218-13 is a discontinuous antigenic determinant. MAb 35-2 showed a surprising reactivity with SDS-denatured RGL (Fig. 4) in contrast to its recognition of the native enzyme. One hypothesis could be that the epitope of mAb 35-2 is hidden in the core of native RGL and becomes accessible in the SDS-denatured enzyme.

FIG. 3. Determination of the immunoreactivity of the various anti-HGL mAbs toward DGL and RGL using an indirect competitive assay. Gastric lipases, at variable concentrations (10^{-10} *M* to 0.2×10^{-6} *M*), were mixed in solution with a fixed amount of each mAb (0.4×10^{-7} *M*). Then the mixtures were incubated with precoated HGL (20 ng/well) in a microtitration plate. The rest of the procedure is the same as that described earlier for ELISA. Binding values were calculated as percentage reduction in absorbance relative to controls without DGL or RGL (100%).

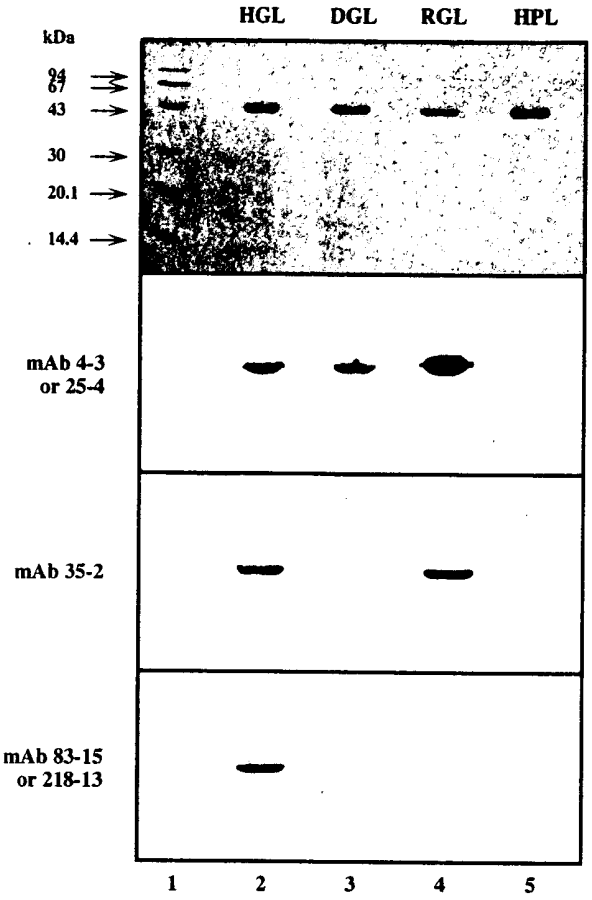
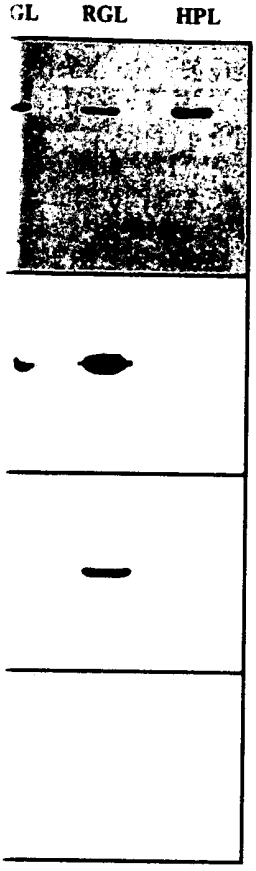


FIG. 4. Upper panel shows SDS–PAGE (12%) patterns of various lipases. Lane 1, molecular mass markers; lane 2, HGL; lane 3, DGL; lane 4, RGL; and lane 5, HPL. Lower panels show immunoblot analysis of the lipases from the upper panel, using anti-HGL mAbs as primary antibody. Alkaline phosphatase-conjugated with goat antimouse IgG (Sigma) was used as secondary antibody.

Immunoaffinity Purification of HGL Using a Monoclonal Antibody

MAb (35-2), with a good affinity to HGL, was produced in large amounts. Purified mAb 35-2 (70 mg) was immobilized on 25 ml of swollen Affi-Gel 10 (Bio-Rad) equilibrated with PBS as described earlier for the case of anti-HGL–pAb. Under these conditions, more than



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HGL, was produced in large
was immobilized on 25 ml of
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these conditions, more than

95% of the initial amount of mAb 35-2 was covalently coupled to the gel.[30]

The first step in the published standard purification of HGL from human gastric juice consisted of a cation exchange chromatography (SP-Sepharose).[9] This step was mostly performed to eliminate components such as mucus and pepsinogens, present in gastric juice, which could affect the immunoaffinity chromatography. Active lipase fractions were concentrated on an Amicon ultrafiltration cell using Diaflo YM-10 membrane, dialyzed against TBS and loaded onto the immobilized mAb column equilibrated with TBS. After a 4-hr incubation period at 4° under rotative agitation (18 runs per minute), the gel was washed several times with TBS until the eluates had no absorbance at 280 nm. Pure HGL was eluted as a single protein peak (see Fig. 5) with 0.2 *M* glycine-HCl buffer (pH 2.2). The eluted fractions contain a single protein band on SDS–PAGE, corresponding to HGL (insert of Fig. 5). The eluted fractions (1 ml) containing HGL activity were pooled, concentrated to about 2 mg/ml, using an Amicon ultrafiltration cell, dialyzed against 20 m*M* MES buffer (pH 6), and stored at −20°. As expected from its acidic stability, the elution from the immunoaffinity column under acidic conditions (pH 2.2) does not alter the enzymatic activity of HGL. This procedure offers the advantage of being rapid and reproducible, and it gives a better overall yield (58%) with a 50-fold purification[30] as compared to the previously described methods using conventional chromatographic steps.[9]

Quantitative ELISA for Measuring HGL in Duodenal Contents[30]

The availability of specific antibodies allowed us to set up a sensitive and specific double sandwich ELISA for measuring the HGL in the duodenal contents in which both HGL and HPL are present.[3] The procedure and the buffers used are those described earlier for the screening of mAbs with minor modifications.

Purified anti-HGL pAb was coated in the PVC microplate (125 ng/well) and used as the captor antibody. After saturation of the wells with saturating buffer, 50 μl of standard solution of HGL (0.1–90 ng/ml) or samples from human duodenal contents, appropriately diluted with the washing buffer, were added to each well. Wells containing buffer without HGL or duodenal contents served as controls. Then 50 μl of anti-HGL mAb-biotin conjugate (1 μg/well) (detector antibody) was added to each well. The biotinylation procedure of mAb is described later. HGL was

[30] M. Aoubala, I. Douchet, R. Laugier, M. Hirn, R. Verger, and A. de Caro, *Biochim. Biophys. Acta* **1169,** 183 (1993).

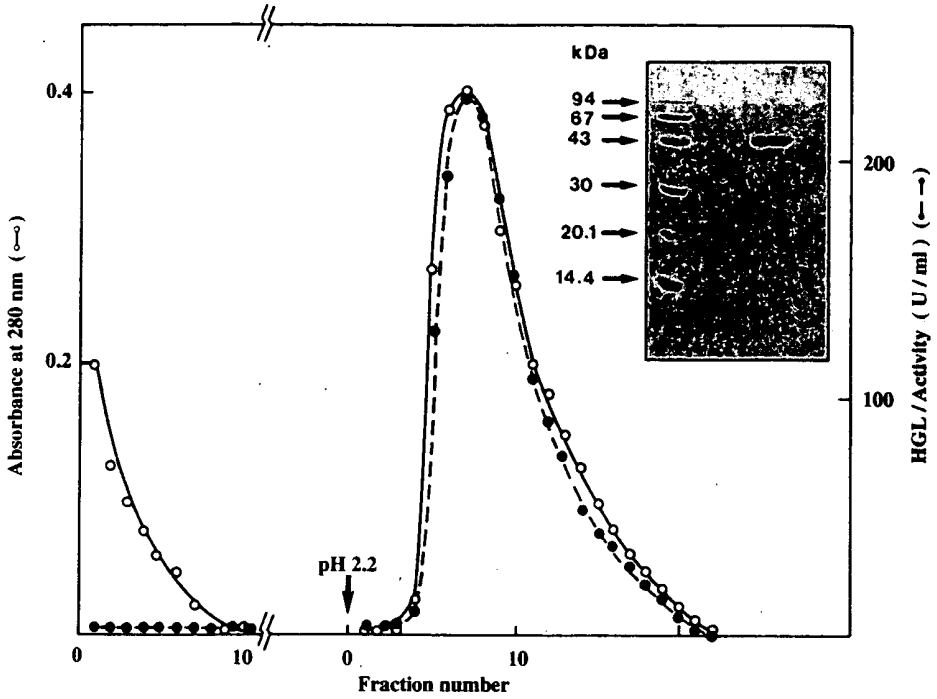
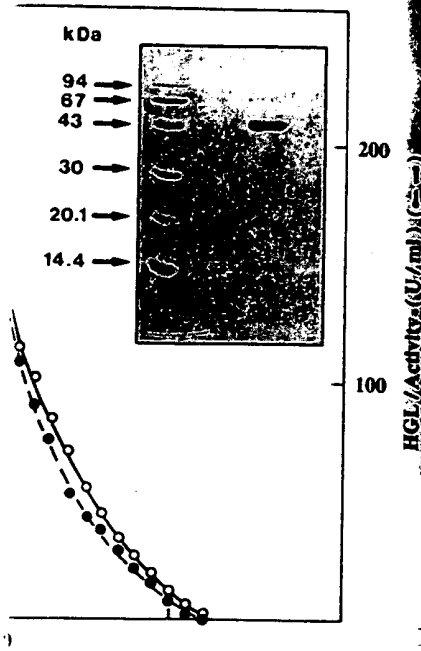


FIG. 5. Purification of HGL by immunoaffinity chromatography using an immobilized mAb (35-2) column. The active fractions obtained by performing SP-Sepharose chromatography were applied to the column and the pure enzyme was eluted with 0.2 *M* glycine-HCl buffer (pH 2.2). The volume of each fraction was 1 ml. Inset: SDS–PAGE analysis of immunoaffinity purified HGL. Left, Pharmacia standard molecular weight markers; right, 5.8 μg of purified HGL.

measured by using a solution of horse radish peroxidase-labeled streptavidine (Immunotech, Marseilles, France) diluted 20,000-fold according to the manufacturer's instructions.

Since the immunoreactivity of anti-HGL pAb and that of five mAbs are known, all the possible combinations were tested in a double sandwich ELISA.[30] The most sensitive ELISAs able to detect HGL concentrations down to 1 ng/ml were those in which the captor antibody (coating antibody) was a pAb and the detector antibody (antibody–biotin conjugate) was mAb 35-2. Various concentrations of captor antibody (pAb) were tested during the coating procedure. At the optimal concentrations of HGL (60 ng/ml) and biotin-mAb 35-2 conjugate (1 μg/ml), a plateau of absorbance at 492 nm was reached at 2.5 μg/ml (final concentration) of captor antibody corresponding to 125 ng/well.



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To study the reproducibility of the assay as well as the interference from the duodenal contents on the ELISA measurements of HGL, two sets of experiments were carried out. First, known concentrations of HGL were prepared in the washing buffer and, second, known concentrations of HGL were added to a complete liquid test meal. Only the ELISA performed with pAb and biotin-labeled mAb 35-2 showed a good correlation between the curves obtained with the known HGL concentrations prepared in the washing buffer and those in a complete liquid test meal. This pair of antibodies (pAb/biotin–mAb 35-2) was therefore selected for measuring the HGL in the duodenal contents. Known amounts of pure HGL were also added to aspirates of duodenal contents containing some endogenous HGL, and then the HGL levels measured by ELISA were compared with those to be expected. The HGL levels measured in these samples ranged between 84 and 110% of the added amounts.[30] In other experiments, various samples from the duodenal contents were assayed using both the ELISA and an enzymatic assay of HGL. A good correlation ($r = 0.95$) was found to exist between the results of the two assays.[30]

We were not able to detect HGL in human sera by using ELISA. Recently, in pre- and postheparin plasma, Bensadoun[31] reported a sensitive double sandwich ELISA for human hepatic lipase (HL) based on the use of two anti-HL mAbs. HL was easily measured in the range of 0.5–5.0 ng/ml.

Interfacial Binding of HGL to Lipid Monolayers, Measured with an ELISA

The ELISA/biotin-streptavidin system has been found to be as sensitive as the use of radiolabeled proteins. Furthermore, biotinylation preserves the biological activities of many proteins. Our aim was to develop a sensitive sandwich ELISA, using the biotin-streptavidin system, and to measure the amount of surface-bound HGL and anti-HGL mAbs adsorbed to monomolecular lipid films.

Protein Biotinylation and Development of an ELISA

The ε-aminocaproic *N*-hydroxysuccinimide ester D-biotin (ACNHS-biotin) was used to couple biotin moieties to ε-amines of HGL lysine residues using a modified procedure previously described by Guesdon *et al.*[32] To obtain the desired stoichiometric ratio of ACNHS-biotin to total lysine residues of the enzyme, a solution of HGL (1 mg/ml) in 20 m*M*

[31] A. Bensadoun, *Methods Enzymol.* **263**, 333 (1996).
[32] J. L. Guesdon, T. Ternynck, and S. Avrameas, *J. Histochem. Cytochem.* **8**, 1131 (1979).

borate buffer (pH 8.0) containing 150 m*M* NaCl was mixed with various volumes of a solution of ACNHS-biotin (10 mg/ml) in dimethyl formamide. The reaction mixture was incubated at room temperature for 20 min under stirring and stopped by adding NH_4Cl (final concentration 0.1 *M*). The mixture was then immediately dialyzed at 4° against 10 m*M* MES buffer (pH 7). The lipolytic activity of HGL was determined both before and after biotin-labeling using the pH-stat and monolayers methods with tributyrin and dicaprin as enzyme substrate, respectively. It was observed for all three ratios used that HGL could be biotin-labeled without any appreciable loss of catalytic activity. Residual activities of 86, 96, and 100% were observed at ratios of ACNHS-biotin to HGL-lysine residues of 0.6, 0.3, and 0.15, respectively. Moreover, biotinylated HGL hydrolyzes dicaprin films similarly as native HGL, indicating that the labeling does not alter the interfacial HGL activity.[33] This is a considerable advantage over previous methods for chemically labeling HGL with radioactive 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), which led to a catalytically inactive lipase.[34]

Two anti-HGL mAbs were also biotin-labeled as previously described[30] and their capacity to immunoreact with HGL was tested using an ELISA test with native HGL directly coated to the wells of the PVC microplates. No decrease in the antigen-binding capacity of biotinylated mAbs 4-3 and 218-13 was observed.

Determination of the Amount of Protein Adsorbed to the Lipid/Water Interface[33]

We used the monomolecular film technique to study the kinetics of hydrolysis catalyzed by biotinylated HGL as well as its binding to dicaprin films. Measurements were performed with the KSV Barostat equipment (KSV–Helsinki, Helsinki, Finland). Readers are referred to the Chapter [13] by Ransac *et al.* in this volume. After recording of the kinetics and film aspiration, the amounts of biotinylated proteins present in the sample containing the film and a corresponding sample (same volume) containing the bulk phase were measured with an ELISA.

All the ELISA tests were performed as described earlier for the measurement of HGL in duodenal contents, with minor modifications as described by Aoubala *et al.*[33] The OD values at 492 nm were plotted as a function of the concentration of biotinylated protein (Fig. 6). A reference

[33] M. Aoubala, M. Ivanova, I. Douchet, A. de Caro, and R. Verger, *Biochemistry* **34,** 10786 (1995).
[34] Y. Gargouri, H. Moreau, G. Piéroni, and R. Verger, *Eur. J. Biochem.* **180,** 367 (1989).
[35] M. W. Bodmer, S. Angal, G. T. Yarranton, T. J. R. Harris, A. Lyons, D. J. King, G. Piéroni, C. Rivière, R. Verger, and P. A. Lowe, *Biochim. Biophys. Acta* **909,** 237 (1987).

M NaCl was mixed with various
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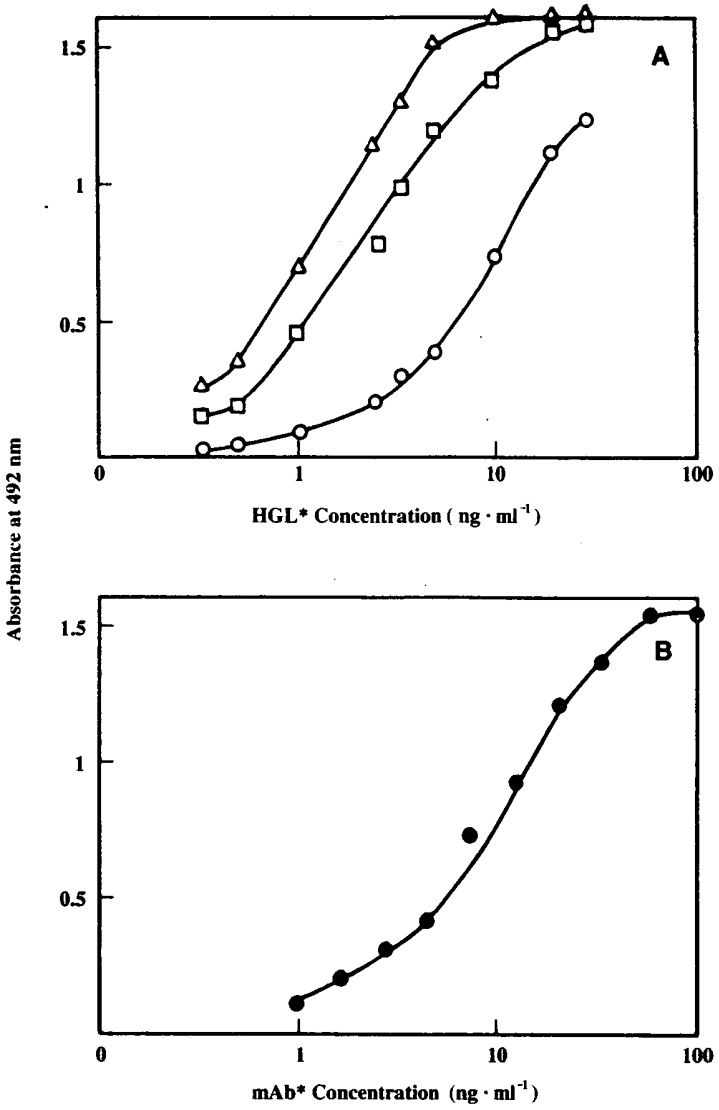

FIG. 6. Reference ELISA curves of (A) biotinylated HGL and (B) biotinylated mAb 4-3. (A) Open triangle, boxes, and circles indicate the titration curves of HGL biotinylated at molar ratios of ACNHS-biotin to HGL lysine amino groups of 0.6, 0.3, and 0.15, respectively. (B) The reference curve of mAb 4-3 biotinylated at one constant molar ratio (0.15) of ACNHS-biotin to mAb lysine amino groups. Biotinylated HGL (HGL*) and biotinylated mAb (mAb*) were dissolved in the washing buffer at concentrations indicated on the abscissa and the immunoreactivity mass of biotinylated proteins was measured with the specific sandwich ELISA.

curve was drawn up for each test and was used to calculate the concentration of biotinylated protein in the aspirated samples recovered from the monomolecular film experiments. Each assay was carried out in duplicate. The detection limit was 25 and 85 pg in the case of HGL and mAb, respectively.[33] The difference between the total amounts of proteins between these two samples was attributed to the surface excess of protein molecules bound to the lipid film.

The volume occupied by the lipid film was not taken into account since it is negligible, with respect to the aspirated subphase. We used the following equation:

$$\Gamma = \frac{[F + B] - [B]}{S} \cdot V_a,$$

where Γ is the surface excess of protein bound to the lipid monolayer, expressed in ng/cm^2; $[F + B]$ is the concentration of protein present in the aspirated film with the aspirated bulk subphase, as determined by ELISA; $[B]$ is the concentration of protein in the bulk sample, also determined by ELISA; V_a is the aspirated volume (ranging from 0.5 to 1.5 ml); and S is the area of the reactional compartment of the trough (39.3 cm^2).

During the monolayer experiments, the validity of the sandwich ELISA for biotinylated HGL was tested in the presence of monomolecular films of either dicaprin or egg phosphatidylcholine (egg PC). The recovery levels of biotinylated HGL injected under lipid monomolecular films were determined after each experiment as

$$\text{Total recovery} \quad [\%] = 100 \cdot \frac{[B] \cdot V_t + \Gamma \cdot S}{T},$$

where $[B]$ is the concentration of biotinylated HGL in the subphase (bulk); V_t is the total volume of the reactional compartment measured after each monolayer experiment (30 ± 2 ml); $\Gamma \cdot S$ is the total amount of biotinylated HGL adsorbed to the monomolecular film, and T is the total amount of biotinylated HGL (200 ng) injected under the monomolecular film. The yields obtained in these conditions were 78 ± 6.2%.

By combining the above sandwich ELISA technique with the monomolecular film technique, it was possible to measure the enzymatic activity of biotinylated HGL on 1,2-didecanoyl-*sn*-glycerol monolayers as well as to determine the corresponding interfacial excess of the enzyme.[33] Figure 7A shows the amounts of biotinylated HGL in excess at the interface. The amounts of HGL present at or close to the interface increased linearly with increasing surface pressures up to 25 mN/m. Furthermore, a decrease in the amount of adsorbed enzyme was observed above this optimal surface pressure value. The lipolytic activity of biotinylated HGL

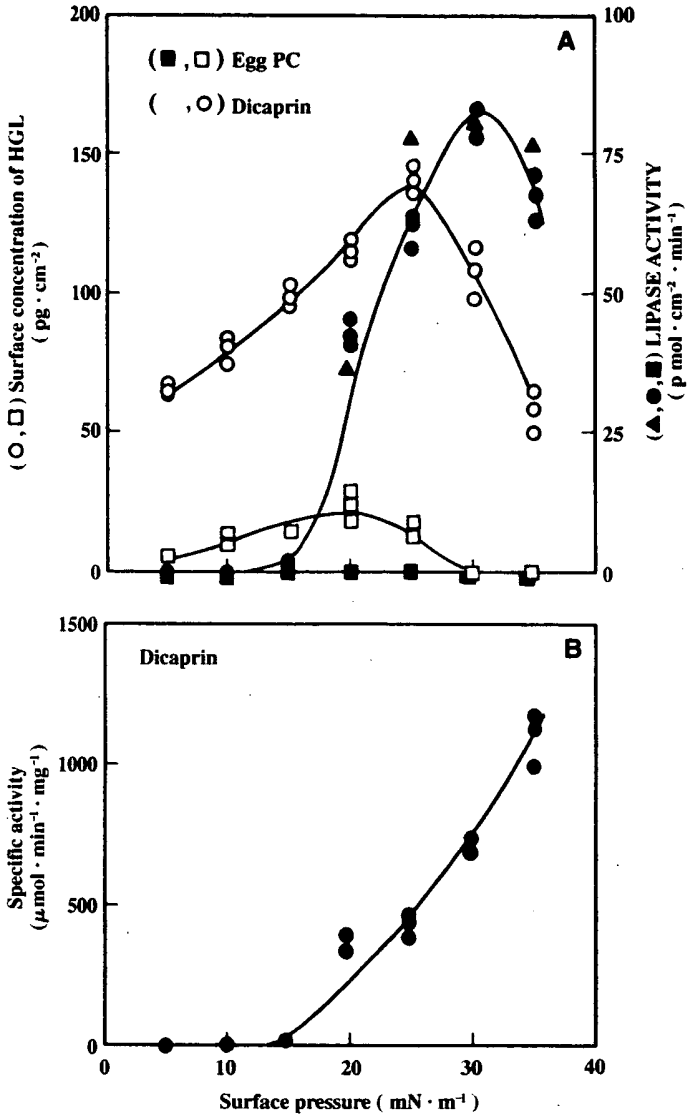

FIG. 7. (A) Variations of the initial velocity of hydrolysis of a dicaprin monolayer with surface pressure (filled circles) by HGL* (200 ng) and (filled triangles) by native nonbiotinylated HGL (200 ng) injected into the subphase (30 ± 2 ml) of a zero-order trough. No enzymatic activity was detected with egg PC films (filled boxes). The interfacial excess of biotinylated HGL was measured 10 min after its injection under a dicaprin (open circles) or egg PC (open boxes) monomolecular film. (B) Variations with surface pressure of the minimal specific activity of biotinylated HGL acting on dicaprin monolayers.

was also measured simultaneously at the corresponding surface pressures. As shown in Fig. 7A, HGL does not significantly hydrolyze dicaprin films at surface pressures of less than 15 mN/m. Above this value the enzymatic activity increases rapidly, reaching a maximum value at 30 mN/m. The ratio of observed enzyme activity to the amount of adsorbed protein, as determined by the specific sandwich ELISA, allows one to calculate the specific activity of the enzyme acting on a monomolecular film of dicaprin. As shown in Fig. 7B, the specific activity increased continuously from 15 up to 35 mN/m. At the latter value, the maximal specific activity reached was 1100 ± 97 U/mg whereas at 15 mN/m, its value was zero. The specific activities determined at 35 mN/m were found to be in the range of the values measured under optimal bulk assay conditions, using tributyrin emulsion as substrate (i.e., 1000 μmol/min per milligram of enzyme).

At a given lipase concentration in the water subphase, the interfacial binding of biotinylated HGL to the nonhydrolyzable egg PC monolayers was found to be 10 times lower than in the case of dicaprin monolayers[33] (Fig. 7A). This observation suggests that the weak affinity of biotinylated HGL for egg PC is probably due to repulsive forces originating from the zwitterionic phospholipid headgroups; whereas the strong interactions observed between the enzyme and the electrically neutral dicaprin monomolecular films in fact reflect the occurrence of strong enzyme/substrate interactions.

It is worth noting that the surface-bound enzyme includes not only those enzyme molecules directly involved in the catalysis but also an unknown amount of protein present close to the monolayer. These enzyme molecules were not necessarily involved in the enzymatic hydrolysis of the film.

Given the low tensioactivity of the mAbs of the IgG isotype,[19] we also investigated the effects of five anti-HGL mAbs (4-3, 25-4, 35-2, 83-15, and 218-13) on the catalytic activity as well as on the interfacial binding of biotinylated HGL to lipid/water interfaces.[33] Four out of these five mAbs (4-3, 25-4, 35-2, and 83-15) were found to reduce significantly the lipolytic activity of HGL. Moreover, three of the four inhibitory mAbs (4-3, 25-4, and 35-2) were found to reduce the specific activity of HGL, whereas mAb 83-15 had no effect on the specific activity. These results clearly indicate that the latter mAb (83-15) complexed with biotinylated HGL mainly affects the binding of the enzyme to the lipid/water interface, whereas the other three inhibitory mAbs (4-3, 25-4, and 35-2) affect both the binding and the catalytic steps of HGL. Therefore, it was possible for the first time to correlate the lipase activity with the surface excess of the enzyme present at the interface.

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Acknowledgments

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